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BASIC SCIENCE

Erectile Dysfunction and Altered Contribution of KCa1.1 and KCa2.3 Channels in the Penile Tissue of Type-2 Diabetic db/db Mice

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Simon Comerma-Steffensen, DVM, MSc, PhD,[1,2] Judit Prat-Duran, MSc,[1] Susie Mogensen, BSc,[1] Rafael Fais, MSc, PhD,[1,3] Estéfano Pinilla, M. Pharma, PhD,[1] and Ulf Simonsen, MD, PhD[1]

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ABSTRACT

Background: Activation of endothelial small conductance calcium-activated K^+ channels (KCa2.3) and intermediate conductance calcium-activated K^+ channels (KCa3.1) leads to vascular relaxation. We found endothelial KCa2.3 down-regulation in the corpus cavernosum diminishes erectile function.

Aim: We hypothesized that in type-2 diabetic mice, the function of KCa2.3 and KCa1.1 channels is impaired in erectile tissue.

Methods: Erectile function was measured, and corpus cavernosum strips were mounted for functional studies and processed for qPCR and immunoblotting.

Outcomes: Effects of type 2 diabetes on erectile function, expression and function of calcium-activated potassium channels.

Results: In anesthetized diabetic db/db mice, erectile function was markedly decreased compared to non-diabetic heterozygous db/+ mice, and the impairment was even more pronounced compared to normal C57BL/6 mice. qPCR revealed KCa2.3 and KCa1.1α channel expressions were upregulated in corpus cavernosum from db/db mice. Immunoblotting showed down-regulation of KCa2.3 channels in the corpus cavernosum from db/db mice. Acetylcholine relaxations were impaired while relaxations induced by the nitric oxide, donor SNP were unaltered in corpus cavernosum from db/db compared to C57BL/6 and db/+ mice. Apamin, a blocker of KCa2 channels, inhibited acetylcholine relaxation in corpus cavernosum from all experimental groups. In the presence of apamin, acetylcholine relaxation was markedly decreased in corpus cavernosum from db/db vs C57BL/6 and db/+ mice. An opener of KCa2 and KCa3.1 channels, NS309, potentiated acetylcholine relaxations in corpus cavernosum from db/+ and db/db mice. Iberiotoxin, a blocker of KCa1.1 channels, inhibited acetylcholine relaxation in corpus cavernosum from db/+ mice, while there was no effect in tissue from db/db mice.

Clinical Translation: Erectile function in diabetic db/db mice was severely affected compared to heterozygous and control mice, findings suggesting the non-diabetic db/+ and diabetic db/db mice for translational purpose can be used for drug testing on, respectively, moderate and severe erectile dysfunction. The altered expressions and impaired acetylcholine relaxation in the presence of apamin compared to C57BL/6 mice may suggest decreased KCa1.1 channel function may underpin impaired endothelium-dependent relaxation and erectile dysfunction in diabetic db/db mice.

Strengths & Limitations: The present study provides a mouse model for type 2 diabetes to test moderate and severe erectile dysfunction drugs. Decreased KCa1.1 channel function contributes to erectile dysfunction, and it is a limitation that it is not supported by electrophysiological measurements.

Conclusion: Our results suggest that the contribution of iberiotoxin-sensitive KCa1.1 channels to relaxation is reduced in the corpus cavernosum, while apamin-sensitive KCa2.3 channels appear upregulated. The impaired KCa1.1 channel function may contribute to the impaired erectile function in diabetic db/db mice. **Comerma-Steffensen S, Prat-Duran J, Mogensen S, et al. Erectile Dysfunction and Altered Contribution of KCa1.1 and KCa2.3 Channels in the Penile Tissue of Type-2 Diabetic db/db Mice. J Sex Med 2022;19:697–710.**

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[1]Department of Biomedicine, Aarhus University, Aarhus, Denmark;

[2]Department of Biomedical Sciences/Animal Physiology, Veterinary Faculty, Central University of Venezuela, Maracay, Aragua, Venezuela;

[3]Pharmacology Department, Ribeirao Preto Medical School, Sao Paulo University, Brasil

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Key Words: Acetylcholine; Diabetes; Erectile Function; Potassium channels; Mice

INTRODUCTION

Erectile dysfunction occurs in 32% of type 1 and 46% of type 2 diabetic men,[1,2] and altered endothelial function appears to be part of the explanation.[3−5] Thus, the vascular endothelium is thought to play an essential role in the mechanism of erection, and increased blood flow during erection may lead to the release of vasorelaxant transmitters such as nitric oxide (NO),[6] prostaglandins, and endothelium-derived hyperpolarization (EDH).[7] Myo-endothelial gap junctions[8], potassium ions[9], products of the cytochrome P450 pathway[10], C-type natriuretic peptide[11], and hydrogen peroxide[12] have been suggested to mediate EDH.

Impairment of endothelial function is thought to be one of the main causes of diabetes-associated erectile dysfunction.[13,14] In penile tissue hyperglycaemia decreases endothelial NO synthase (eNOS) activity[15] and phosphorylation[16], and it leads to eNOS uncoupling.[16] Hyperglycaemia is associated with increased formation of reactive oxygen species, which act as NO scavengers and therefore reduce NO bioavailability.[17,18] Vasorelaxant responses mediated by EDH are also reduced in penile arteries from Zucker diabetic fatty (ZDF) rat[7] and diabetic men with erectile dysfunction.[19] Diabetes seems to impair both NO and EDH-mediated vasodilation, and reactivation of both pathways has also been suggested to regain penile erectile function in streptozotocin-induced diabetic rats.[20]

Calcium-activated potassium channels play a role in the neurogenic phase as well as in the endothelium-dependent relaxations of erectile tissue during erection (Figure 1). Thus, large-conductance calcium-activated K^+ channels (KCa1.1) in the smooth muscle were found to mediate the neurogenic and NO-induced relaxations in horse penile arteries[21] and the endothelium-dependent relaxations in rat penile arteries both in response to acetylcholine and increased flow.[7,22] In mice lacking KCa1.1 channels, erectile dysfunction was reported.[23] Moreover, openers of KCa1.1 channels were found to relax erectile tissue[24−26] and penile arteries from man[19,26,27], and infusion of an opener of KCa1.1 channels enhances the erectile responses in rats.[26] In addition to KCa1.1 channels, endothelial KCa2.3 channels were suggested to be involved in endothelium-dependent vasodilatation in human and rat intracavernous penile arteries and relaxation of rat corpus cavernosum.[7,11,28] Infusion of openers of endothelial KCa2.x and KCa3.1 channels, NS309 and NS4591, enhanced erectile function in healthy rats[28], and in small mesenteric arteries from Zucker diabetic fatty rats relaxations induced by the endothelium-dependent vasodilator, acetylcholine can be restored by treatment with an opener of KCa2.x and KCa3.1 channels, NS309.[29] Expression of KCa2.3 and KCa3.1 channels was reported to be reduced in corpus cavernosum tissue of streptozocin-induced type 1 diabetes in rats.[30] Still, it is unclear whether the contribution of these channels is altered in type 2 diabetes.

Diabetic (db/db) mice develop obesity, insulin resistance, hyperglycaemia, and hyperlipidemia similar to that seen in human type 2 diabetes due to a spontaneous mutation in the leptin receptor gene.[30] In the present study, we hypothesized that in type-2 diabetes, the function of KCa2.3 and KCa1.1 channels is impaired in erectile tissue. To address the hypothesis, erectile function was investigated in db/db mice, and corpus cavernosum strips were mounted for functional studies, and expression of KCa channels was examined by qPCR and immunoblotting. To control for the effect of leptin receptor downregulation on erectile and endothelial function in the non-diabetic db/+ mice, we also investigated normal C57BL/6 mice. The endothelial layer plays an important role for maintaining erectile function, and the endothelium-dependent relaxation was investigated followed by examination of the effect of blocking KCa2 channels and KCa1.1 channels, respectively, with apamin and iberiotoxin to elucidate alterations in the function of endothelial KCa2.3 and smooth muscle KCa1.1 channels.

MATERIALS AND METHODS

Animals

Male 14−17 week old db/db mice (C57BLKS/J-leprdb/leprdb) (n = 27) and age- and sex-matched db/+ littermate controls (C57BLKS/J-leprdb/leprdb+) (n = 25), and normal C57BL/6 mice (n = 15) were purchased from Taconic Europe (Ry, Denmark). Age-matched male C57BL/6, db/+, and db/db mice were included for checking normal erectile function. The mice were housed in the animal facility in 365 × 207 × 140 mm cages with standard wood bedding and space for 3 to 4 mice, and they had free access to water and laboratory diet (Brogaarden, Middelfart, Denmark). They were allowed to acclimatize for at least 1 week before experimentation. The mice had a 12-hour light/dark cycle, and to avoid the influence of torpor,[31,32] the mice were allocated to investigation early in the light period. Blood glucose was measured weekly in non-fasting animals using arterial tail blood with a Contour Blood Glucose Monitoring System (Bayer, Denmark, Copenhagen S). The different appearance in size of db/+ vs db/db mice made it impossible to blind the observer for the in vivo experiments. All animal care and experimental protocols in this study were conducted under the supervision of a veterinarian and in accordance with the Danish legislation of animal use for scientific procedures as described in the "Animal Testing Act" (Consolidation Act No. 726 of 9 September 1993 as amended by Act No.

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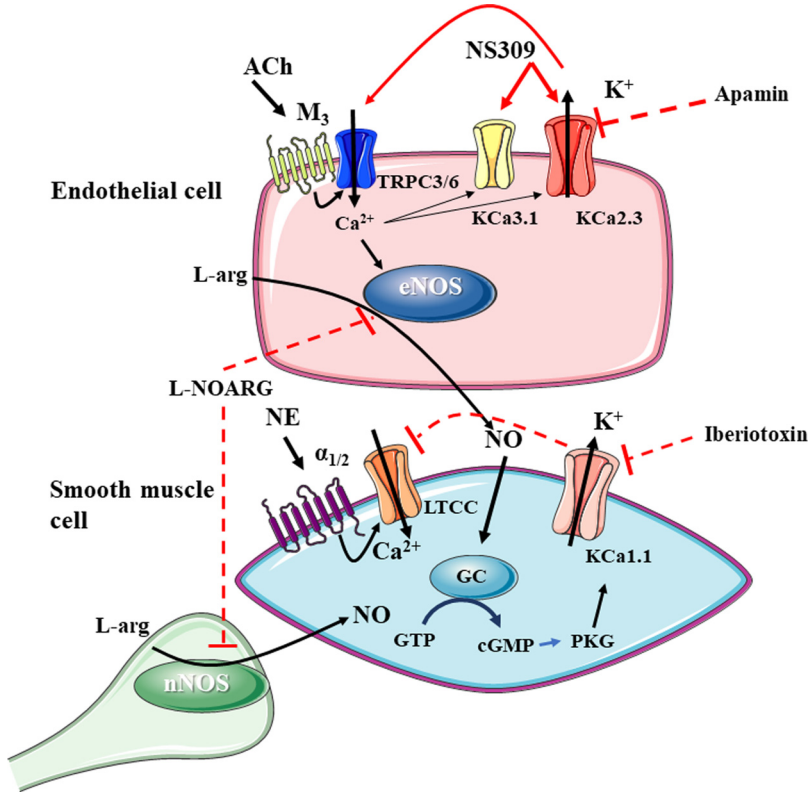

Figure 1. Role of calcium-activated potassium channels in corpus cavernosum contractility. Calcium-activated potassium channels of small (KCa2.1) and intermediate (KCa3.1) conductance are expressed in the endothelial cell layer, while calcium-activated potassium channels of large-conductance (KCa1.1) are expressed in the smooth muscle cells. ACh, acetylcholine; M3, muscarinic M3 receptor; TRPC3/6, cation transient receptor potential channels type 3 and 6. NE, norepinephrine activates α1 and α2-adrenceptors in the smooth muscle cell layer. LTCC, voltage-gated L-type calcium channel. Broken red lines indicate inhibition, while full red line indicate potentiation and/or activation. Figure is available in color online at www.jsm.jsexmed.org.

1081 of 20 December 1995) and in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/E.U. for animal experiments and approved by the Danish Animal Experiments Inspectorate (permission 2014-15-2934-01059 and 2019-15-0201-00009). The Danish Animal Testing Act thoroughly and extensively covers the requirements included in the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Health Institute. The animal studies are reported in compliance with the ARRIVE guidelines.[33,34]

Measurement of Erectile Function

Mean arterial blood pressure (MAP) and intracavernous blood pressure (ICP) was measured using catheters placed with PE50-PE10 and PE50-25G needle, respectively, in the carotid artery and corpus cavernosum as previously described.[35] Maximal stimulation (6V, 1ms, 10Hz, 60s) was applied to check maximal erectile function at the beginning of each experiment, before incremental frequencies (2, 4, 8, and 16Hz) were applied at 1.5, 3, and 6V. At the end of the experiment, the maximal response was repeated to ensure that the cavernous nerve was intact and erectile function was maintained.

Isometric Tension Recording in Isolated Corpus Cavernosum

After dissection of corpus cavernosum as previously reported,[26,35] the strips were mounted between 2 wire clamps with one clamp connected to an isometric transducer (Danish Myo Technology), and immersed in 10 mL of physiological salt solution (PSS), bubbled with a gas mix (20% O_2, 5% CO_2 and 75% N_2) while kept at 37°C during the whole experiment.[35] For strips from db/+ and db/db mice length−contraction curves were constructed with norepinephrine (3 μM) and then acetylcholine (ACh, 1 μM) to set an optimal contraction and relaxation length (Figure S1).

After stable basal tension, corpus cavernosum strips were activated with a high potassium salt solution (125 mM KPSS). Afterward, endothelial function was assessed with norepinephrine (3 μM), and ACh (1 μM). Concentration-response curves (0.001 −0.3 μM) were constructed for norepinephrine. The preparations were contracted with norepinephrine to 80% of the maximum response and concentration-response curves constructed for ACh, a muscarinic receptor agonist, NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime), a KCa2 and KCa3.1 opener, and sodium nitroprusside (SNP) a NO donor (0.001−0.3 μM).

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To investigate the involvement of KCa2.1-3 channels in acetylcholine relaxation, the preparations were incubated with NS309 (5×10^{-7} M) or apamin (10^{-7} M) prior to the construction of concentration-response curves for acetylcholine.

qPCR

Corpus cavernosum tissue was stored in RNA later (Sigma-Aldrich) until extraction and purification of total RNA were performed using the RNeasy Mini Plus Kit (Qiagen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Life Technologies).

The qPCR was performed in a MX3005 qPCR system (Agilent Technologies, Glostrup, Denmark). The samples were run for a 40 cycles protocol. Ct-values for the gene of interest were normalized against Ct values for the housekeeping gene (GAPDH), after quantification with the program MxPro v.4.10. (Stratagene, Agilent Technologies). Values are expressed as a ratio of GAPDH. For genotyping of the mice, conventional PCR was performed in a Peqstar thermal cycler (Peqlab). The protocol followed a "hot-start" procedure and thermal cycling conditions as explained in the supplemental methods.

We have previously reported the sequences of the probes and primers applied in the present study (see Supplementary Table S1 in[36]).

Immunoblotting

Corpus cavernosum and aorta from db/+ mice, diabetic db/db mice, and WT mice were snap-frozen and kept at -80°C in different quantities for KCa evaluation. Protein was extracted, quantified, and mixed in sample buffer before being separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). The following protein amounts were used for detection: 5 μg corpus cavernosum for KCa2.3 (sc-28621), KCa3.1, KCa1.1α (-alpha-subunit) (APC-107), and KCa1.1β1 (beta-1-subunit) (ab3587); cerebellum and liver 8 μg for KCa1.1β1. Samples were incubated with the indicated antibodies, all raised in rabbits: KCa2.3 (1:200, sc-28621, Santa Cruz Biotechnologies, Heidelberg, Germany), KCa1.1α (1:100, APC-107, Alomone Labs, Jerusalem, Israel), KCa1.1β1 (1:250, ab3587, AbCam, Cambridge, United Kingdom); and housekeeping protein, pan-actin (1:1000, Cat# 4968, Cell Signaling Technology, MA, USA). Membranes were then incubated with a secondary anti-rabbit IgG (1:4000) and processed with an ECL-Plus kit (General Electric "GE" Health care). I-block 0.3% was used as a blocking ingredient. The intensity was quantified by GeneTools (Syngene) and expressed relative to total protein in the membrane. Concerns have been raised concerning the KCa1.1β1 antibody,[37] and therefore, experiments were conducted with a blocking peptide, Maxi Potassium channel beta/KCNMB1 peptide (ab5023, Abcam) and showed that bands were specific at 32 and 115 KDa, and only these bands were quantified, although db/+ and db/db mice showed additional ones. For further details, please see the file with the supplementary full blots.

Statistical Analysis

Statistical comparisons were performed using Graphpad Prism version 9.2 (GraphPad Software). Statistical parametric assumptions were determined by q-q plots and multiple tests. If not the following normality, logarithmic transformation was performed, and values are presented as confidence intervals on the following way medians (quartiles 25−75%). Parametric values are presented as means ± SEM QPCR and immunoblotting results were compared with Student's *t*-test or in the case of 3 groups with 1-way ANOVA followed by Tukey test for multiple comparisons. Norepinephrine-induced-contractions were expressed as force gram (g) and milligram (mg) of corpus cavernosum dry weight (g/mg). The responses to ACh, NS309, or SNP were expressed as a percentage of relaxation of norepinephrine-(3 μM)-contracted strips. Concentration-response curves were compared using 2-way ANOVA followed by a Tukey post-test or a *t*-test when a single concentration between 2 groups was compared. When the response of a single concentration was examined, with more than 2 groups, one-way ANOVA followed by Tukey post-test for multiple comparisons was used. Erectile function was analyzed as the ratio of peak ICP (PICP)(mmHg)/mean arterial pressure (MAP) (mmHg)x100. For each frequency, 2-way ANOVA with a Tukey test for multiple comparisons were used. Significance was accepted at $P \leq .05$.

RESULTS

Measurements of Erectile Function

The experimental weight and blood glucose were significantly higher in db/db compared to db/+ mice and C57BL/6 (Table 1). In recent studies, we have found that blood pressure and heart rate are unaltered.[38]

Stimulation of the cavernous nerve induced frequency-dependent increases in intracavernous pressure in normal C57BL/6 mice, normoglycaemic db/+, and diabetic db/db mice (Figure 2A, B). The erectile function measured as PICP/MAP showed that the responses in diabetic db/db mice were consistently lower than in normoglycaemic db/+ mice (Figure 2B), and the responses in both db/+ and db/db mice were markedly lower than in normal mice (Figure 2B). These findings suggest normoglycaemic db/+ mice have moderate erectile dysfunction and

Table 1. Body weight and blood glucose levels in C57BL/6, db/+, and db/db mice

Mouse	n	Weight (g)	Blood glucose (mmol/L)
C57BL/6	7	29.5 ± 0.7	6.6 (6.4−7.3)
db/+	23	29.6 ± 0.5	6.9 (5.9−7.8)
db/db	27	47.2 ± 1.4*,†	28.8 (25.6−33.3)*#

Weights are represented as means±SEM and blood glucose as confidence intervals means (quartiles 25-75%).
Number of animals, n. One-Way ANOVA with Tukey Post-hoc test.
*$P < .05$ db/db vs C57BL/6 and
†$P < .05$ db/db vs db/+.

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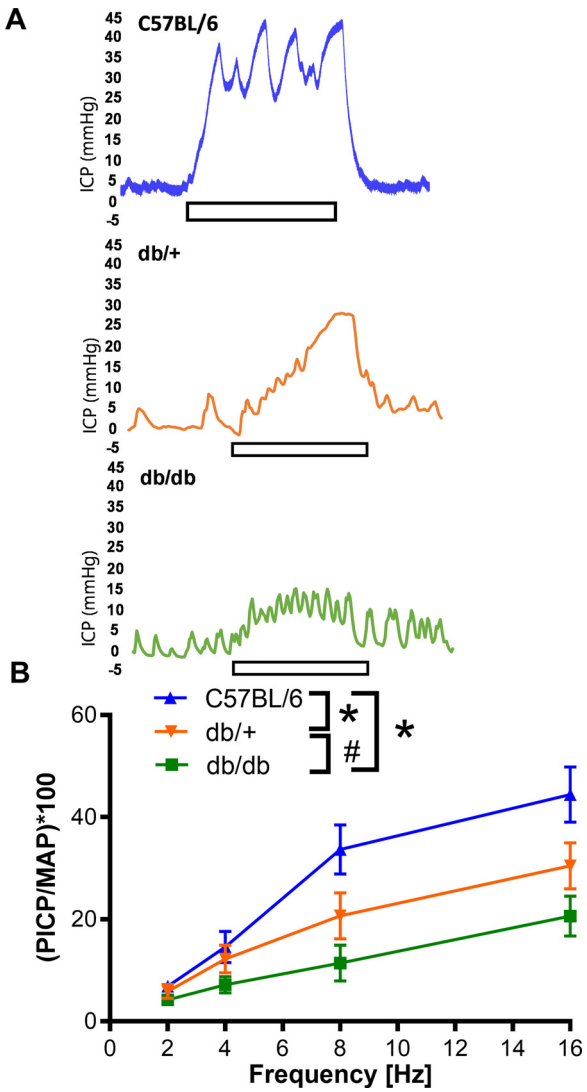

Figure 2. Impaired erectile function in diabetic db/db mice. (A) Original traces showing intracavernosal pressure increase (ICP) in a C57BL/6 mouse (upper trace), a heterozygous non-diabetic db/+ mouse (middle trace), and a diabetic db/db mouse (lower trace). The vertical scale corresponds to intracavernous pressure (ICP, mm Hg). The horizontal bar corresponds to 60 s electric stimulation (6V, 16 Hz) of the cavernous nerve. (B) Average erectile responses in normal C57BL/6 mice (n = 6), non-diabetic db/+ mice (n = 5), and diabetic db/db mice (n = 7). The peak intracavernous pressure (PICP) is normalized to the mean arterial pressure (MAP). Data are means ± SEM * $P < .05$ vs C57BL/6 mice, #$P < .05$ vs db/+ mice. Figure is available in color online at www.jsm.jsexmed.org.

diabetic db/db mice have severe erectile dysfunction compared to normal C57BL/6 mice.

Expression of Calcium-Activated Potassium Channels in Corpus Cavernosum

qPCR revealed that KCa2.3 and KCa1.1 subunits were the most expressed at the gene level of the KCa channel family in the corpus cavernosum (Figure 3). While expression of KCa2.1 was unaltered, the expression of KCa2.2 channels was decreased in corpus cavernosum from both db/+ and db/db mice compared to C57BL/6 mice (Figure 3A, B). Compared to normal C57BL/6 mice, expressions of KCa2.3 and KCa3.1 channels subunit were significantly increased in the corpus cavernosum of diabetic db/db mice compared to expression in the corpus cavernosum of normoglycaemic db/+ and C57BL/6 mice (Figure 3C-D). The gene expression of the KCa1.1α subunit was also upregulated in the corpus cavernosum of db/db compared to C57BL/6 mice (Figure 3E), while expression of the KCa1.1β subunits was unaltered. These findings suggest that at the gene level, the expression of the most expressed channels, KCa2.3 and KCa1.1, are upregulated in the corpus cavernosum of diabetic db/db mice.

Upregulation at gene level may suggest compensatory mechanism. Immunoblotting revealed that the KCa2.3 protein was markedly downregulated in the corpus cavernosum from db/+ and db/db mice, though only a significant decrease was observed in db/+ erectile tissue compared to C57BL/6 mice (Figure 4A). Protein expression of KCa1.1α and KCa1.1β subunits were unaltered in the corpus cavernosum of both db/+ and db/db mice compared to C57BL/6 mice (Figure 4B, C). The decreased of protein expression of KCa2.3 channels may suggest a compensatory upregulation of mRNA expression of these channels.

Functional Studies in Corpus Cavernosum Strips

Length-tension curves were conducted in corpus cavernosum from normoglycaemic db/+ and diabetic db/db mice and revealed the optimal passive tension of the preparations is 1.8 mN (Supplementary Figure S1). In these conditions, the contraction induced by 125 mM KPSS was significantly increased in corpus cavernosum from diabetic db/db animals compared to db/+ and normal mice (Table 2).

Norepinephrine induced concentration-dependent contractions that were rightward shifted in corpus cavernosum from diabetic db/db mice compared to C57BL/6 mice (Supplementary Figure S2, Table 2). Contractions induced by the α_1-adrenoceptor agonist, phenylephrine, were increased in corpus cavernosum from diabetic db/db mice (Supplementary Figure S2B), while contractions induced by the α_2-adrenoceptor agonist, clonidine, were significantly decreased in corpus cavernosum from diabetic db/db and normoglycaemic db/+ mice compared to normal C57BL/6 mice (Supplementary Figure S2C, Table 2). These findings suggest the altered norepinephrine responses in corpus cavernosum from diabetic db/db mice can be ascribed to changed activation of both α_1-and α_2-adrenoceptors, although we also observed relaxations induced by the β-adrenoceptor agonist, isoprenaline, were impaired in corpus cavernosum from diabetic db/db mice (Supplementary Figure S2D).

Norepinephrine contractions induced for construction of the relaxation curves were comparable in corpus cavernosum from the 3 experimental groups (Table S1). Thus, in norepinephrine-

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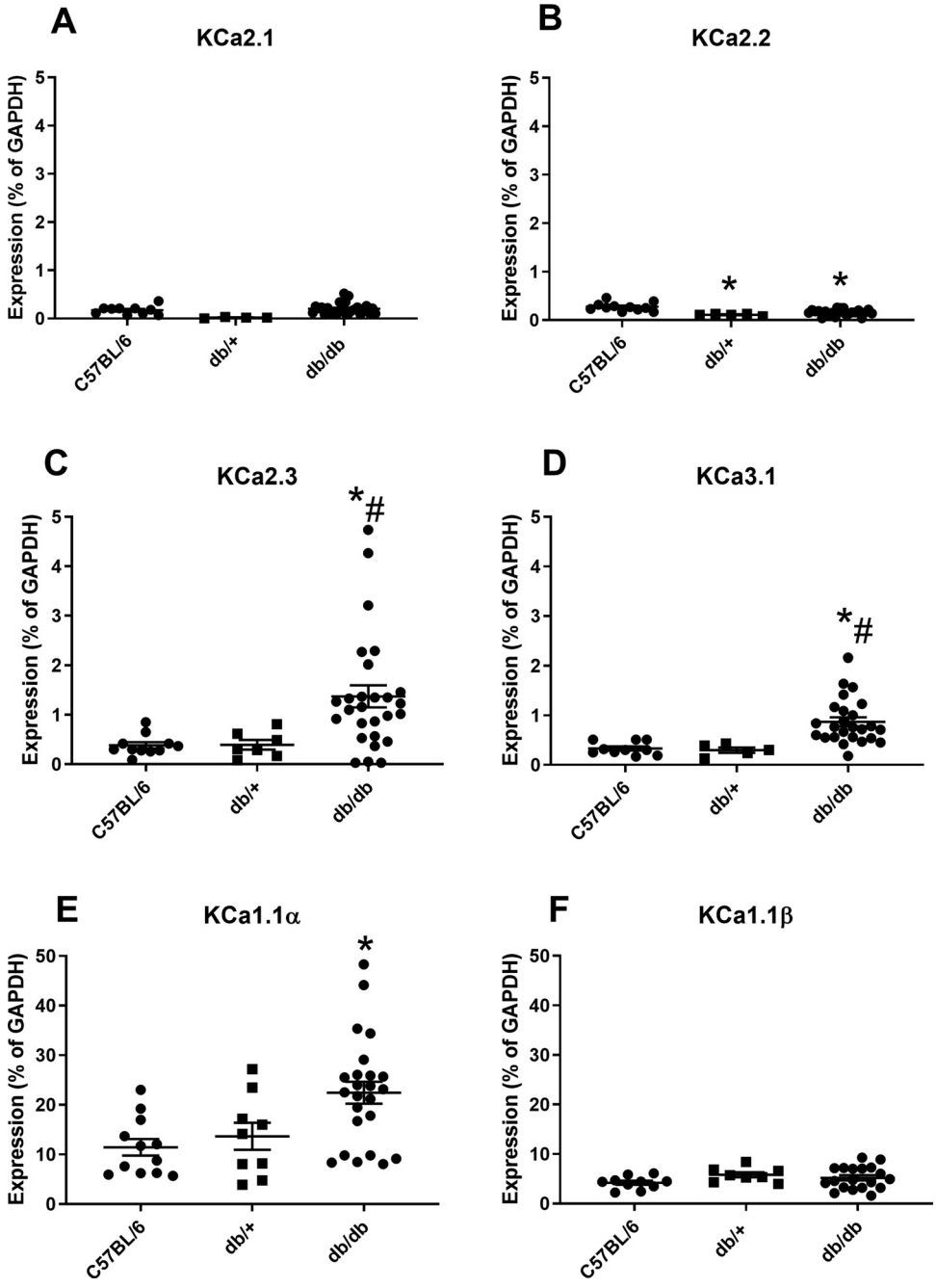

Figure 3. KCa channel expression in channels in corpus cavernosum of control C57BL/6 mice, db/+ and diabetic db/db mice. qPCR showing expression of calcium-activated potassium channels in corpus cavernosum: (A) KCa2.1, (B) KCa2.2, (C) KCa2.3, (D) KCa 3.1, (E) KCa1.1α, and (F) KCa1.1β. Data are means ± SEM, where each symbol corresponds to an animal in each group. Please note the different scale sizes. The expression is shown as percentage of the housekeeping gene GAPDH. * $P \leq .05$ vs C57BL/6 mice, # $P < .05$ vs normoglycaemic (db/+) mice. Compared with one-way ANOVA followed by a Tukey multiple comparisons test. Figure is available in color online at www.jsm.jsexmed.org.

contracted corpus cavernosum strips, acetylcholine-induced concentration-dependent relaxations were unaltered in corpus cavernosum from db/+ compared with the response in those from diabetic db/db mice (Figure 5A). However, the relaxation curves for acetylcholine in the corpus cavernosum from db/db mice were significantly reduced compared to the acetylcholine response in preparations from C57BL/6 mice. An inhibitor of NO synthase, L-NOARG (10^{-4} M), abolished acetylcholine relaxation in corpus cavernosum from db/+ and db/db mice (Supplementary Figure S3A).

Studies in phenylephrine-contracted corpus cavernosum strips revealed impaired acetylcholine relaxations in preparations from diabetic db/db mice compared to normoglycaemic db/+ mice and normal C57BL/6 mice (Figure S4).

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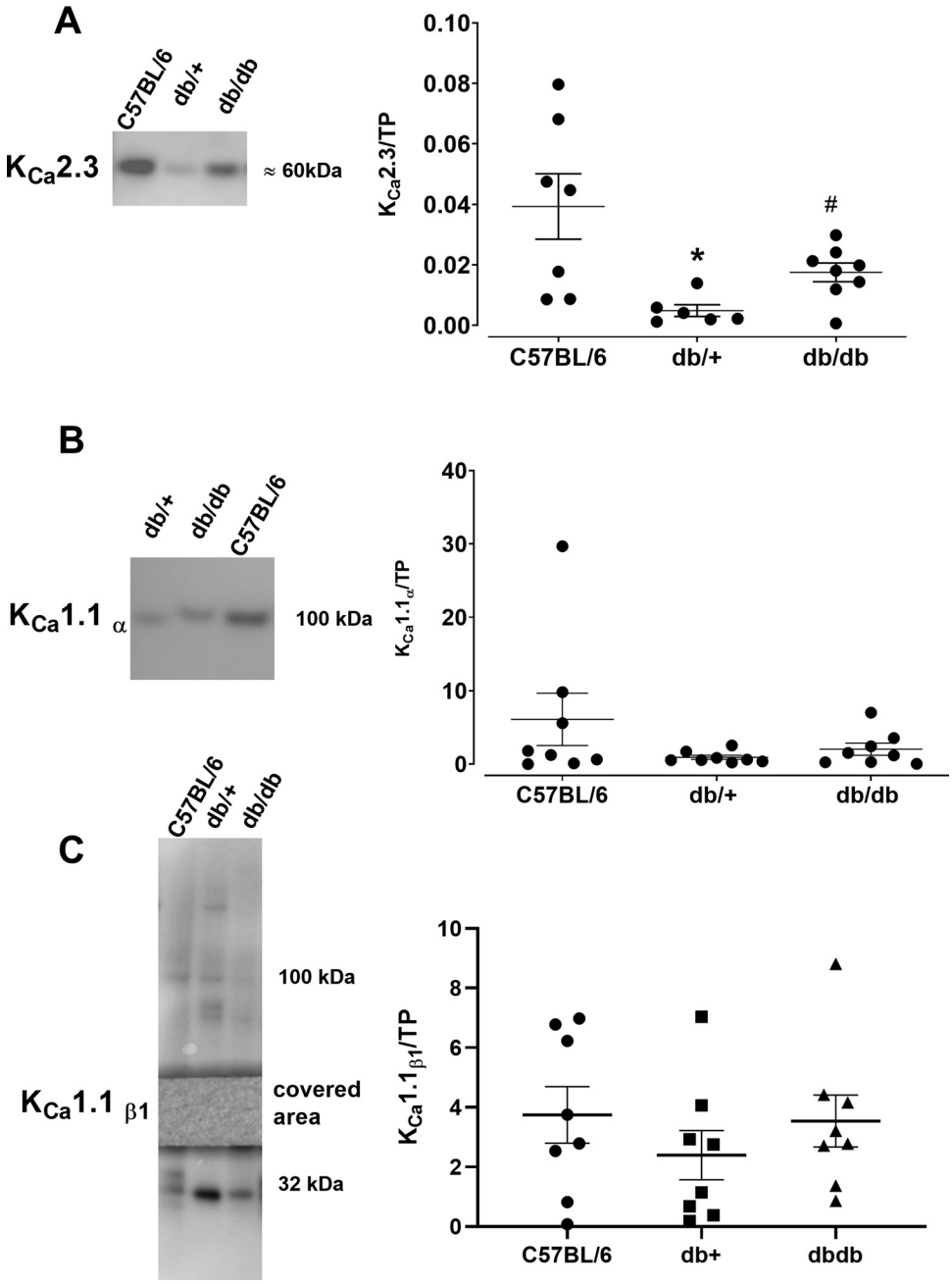

Figure 4. Immunoblotting showing expression of KCa2.3 and KCa1.1 in corpus cavernosum of C57BL6, db/+ and diabetic db/db mice. Immunoblotting shows the expression of the calcium-activated potassium channels in the corpus cavernosum: (A) KCa2.3, and the subunits (B) KCa1.1α, and (C) KCa1.1β. The expression is a ratio of total protein bands in the gel. In Figure 3C, an area of the blot was covered to provide more prominent contrast and allowing quantification at the band at 32 kDa. * $P \leq .05$ vs C57BL/6 mice, # $P < .05$ vs normoglycaemic (db/+) mice. Compared with one-way ANOVA followed by a Tukey multiple comparisons test. Figure is available in color online at www.jsm.jsexmed.org.

In norepinephrine-contracted preparations, the relaxations induced by the NO donor, sodium nitroprusside (SNP), were unaltered in corpus cavernosum from diabetic db/db compared to normoglycaemic db/+ and C57BL/6 mice (Figure 5B) and were blocked to the same degree in the presence of an inhibitor of guanylate cyclase, ODQ (3 μM) (Supplementary Figure S3B).

Functional Role of Calcium-Activated K Channels in Corpus Cavernosum From Diabetic Mice

In norepinephrine-contracted preparations incubated with the SK_{Ca} blocker apamin, acetylcholine relaxations were significantly decreased compared with the control curves for acetylcholine in corpus cavernosum strips from C57BL/6, db/+, and diabetic db/db mice (Figure 6A–C). In the presence of apamin,

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Table 2. Weight of corpus cavernosum preparations and contractions induced by high potassium saline (KPPS) and by norepinephrine in corpus cavernosum from C57BL/6, db/+, and db/db mice

Mouse	Corpus cavernosum (mg)	KPSS (g/mg)	Norepinephrine	
			-log (EC_{50})	Max. contraction (g/mg)
C57BL/6	0.46 ± 0.04 (15)	0.45 ± 0.03 (15)	6.26 ± 0.14 (8)	0.54 ± 0.04 (8)
db/+	0.56 ± 0.03 (23)	0.44 ± 0.03 (23)	5.69 ± 0.10 (16)*	0.48 ± 0.03 (16)
db/db	0.46 ± 0.11 (15)	0.71 ± 0.07 (15)*,†	5.79 ± 0.08 (13)*	0.60 ± 0.05 (13)#

Data are means ± SEM of number of animals (n).
EC_{50} is the concentration causing half-maximal contraction.
One-Way ANOVA with Tukey Post-hoc test.
*$P < .05$ db/db vs C57BL/6 and
†$P < .05$ db/db vs db/+.

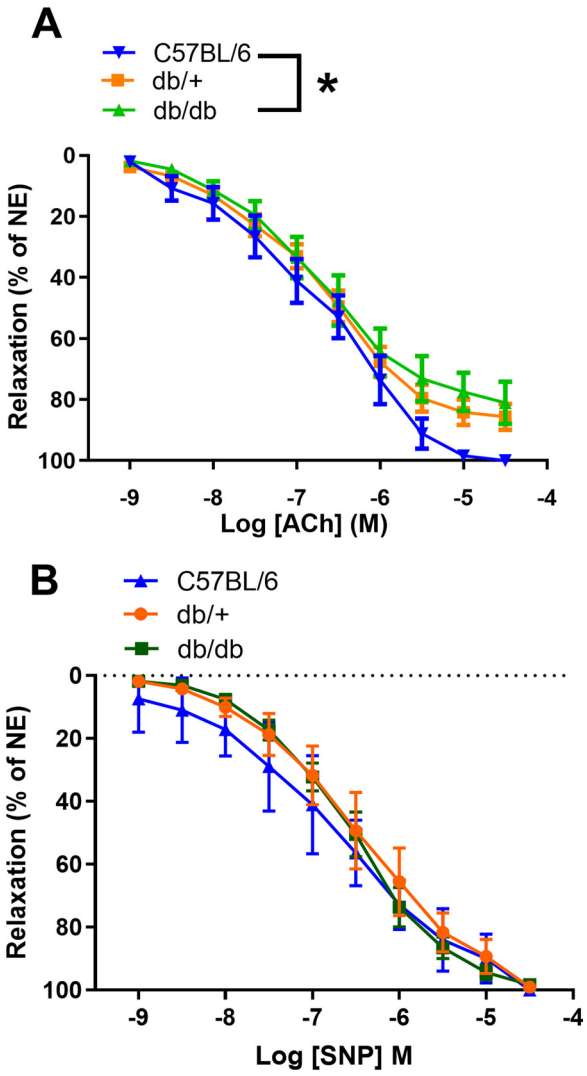

Figure 5. Acetylcholine (ACh) and sodium nitroprusside (SNP) relaxations in corpus cavernosum of diabetic mice. Preparations were contracted with norepinephrine and concentration–response curves performed for (A) ACh (1 nM–30 μM) in corpus cavernosum from normal C57BL/6 (n = 8), non-diabetic db/+ (n = 12), and diabetic db/db (n = 9) mice. (B) Concentration–response curves for SNP (1 nM–30 μM) in corpus cavernosum from C57BL/6 (n = 7), db/+ (n = 6), and db/db (n = 6) mice. * $P < .05$ vs normal C57BL/6 mice, #$P < .05$ vs db/+ mice. Figure is available in color online at www.jsm.jsexmed.org.

concentration-response curves for acetylcholine were significantly reduced in the corpus cavernosum from diabetic db/db compared to normoglycaemic db/+ and C57BL/6 mice (Figure 6D). These findings suggest that a mechanism unrelated to apamin-sensitive channels contributes to reduced acetylcholine relaxation. The apamin-sensitive KCa2 channels appear to have a larger contribution to acetylcholine relaxation in the corpus cavernosum from diabetic db/db mice. This was further supported by subtracting the log area under the concentration curves for acetylcholine in the absence and the presence of apamin, showing a larger reduction of area under the curve in db/db mice vs C57BL/6 mice (insert in Figure 6D).

Previous studies have observed that incubation with 0.5 μM NS309 leftward shifted concentration-response curves for acetylcholine in norepinephrine-contracted corpus cavernosum strips from wild-type mice.[36] In the present study, NS309 had the same effect on acetylcholine relaxations in corpus cavernosum from db/+ and db/db mice (Supplementary Figure S4).

The BK_{Ca} blocker, iberiotoxin inhibited acetylcholine relaxations in corpus cavernosum from db/+ mice, while there was no effect in corpus cavernosum from db/db mice (Figure 7A–C). In the presence of iberiotoxin, there was no change in acetylcholine relaxation of corpus cavernosum from db/db vs db/+ and C57BL/6 mice (Figure 7D). These findings suggest the functional contribution of KCa1.1 channels to acetylcholine relaxation is reduced in the corpus cavernosum from diabetic db/db mice vs normoglycaemic db/+ mice.

DISCUSSION

In the present study, erectile function in diabetic db/db mice was severely affected compared to heterozygous and control mice, findings suggesting the non-diabetic db/+ and diabetic db/db mice for translational purpose can be used for drug testing on, respectively, moderate and severe erectile dysfunction. The decreased function of iberiotoxin-sensitive KCa1.1 channels compared to db/+ mice may underpin the decreased endothelium-dependent relaxation and erectile dysfunction in diabetic db/db mice.

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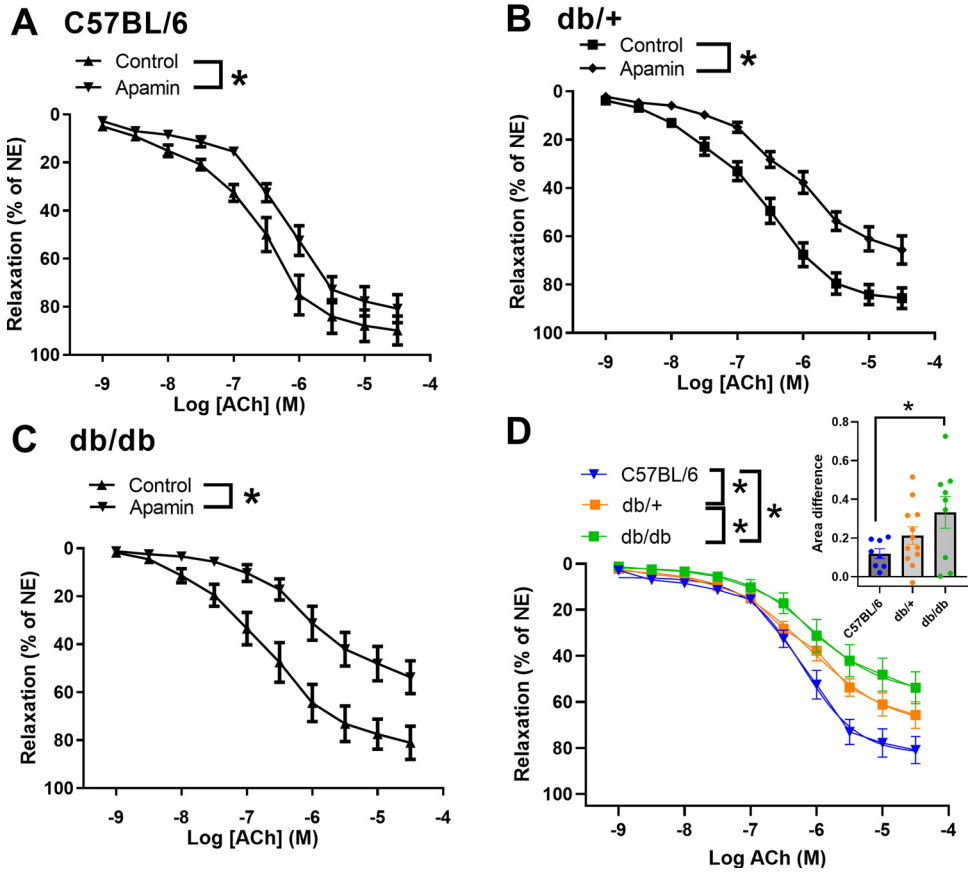

Figure 6. The function of apamin-sensitive KCa2 channels is increased in corpus cavernosum from diabetic animals. Concentration-response curves for ACh in the absence and the presence of apamin (0.5μM) in corpus cavernosum from (A) C57BL/6 (n = 8), (B) db/+ mice (n = 12), (C) diabetic db/db mice (n = 9). Results are means ± SEM * $P < .05$ vs control curve. (D) Concentration-response curves for ACh obtained the presence of apamin in corpus cavernosum from C57BL/6 mice, db/+ mice, and diabetic db/db mice. Insert shows the difference in log area under the concentration-response curves of ACh in the absence and the presence of apamin. #$P < .05$ vs curve obtained in corpus cavernosum of C57BL/6 mice. Figure is available in color online at www.jsm.jsexmed.org.

There are few studies of erectile function in type 2 diabetic animal models compared to control animals. Obese-diabetic Zucker rats were found to have erectile dysfunction compared to Sprague Dawley control rats,[39] while we did not find erectile dysfunction in diabetic compared to the non-diabetic Zucker rats,[7] suggesting obesity and hyperlipidemia rather than high plasma glucose play a role for the erectile dysfunction. The first in vivo mouse model of type 2 diabetic erectile dysfunction used the leptin receptor mutated db/db and wild-type control BKS mouse, and the authors found erectile function was significantly decreased in db/db vs BKS mice in a manner consistent with impairments in veno-occlusive ability and decreased inflow.[40] To control for the influence of down-regulation of the leptin receptor, we in the present study included both wild-type C57BL/6 mice, non-diabetic heterozygous db/+, and diabetic db/db mice. In db/db mice, erectile function was markedly decreased compared to non-diabetic heterozygous db/+ mice, and the impairment was even more pronounced compared to normal C57BL/6 mice. These findings suggest that leptin receptor down-regulation leads to erectile dysfunction and that the presence of high plasma glucose further aggravates the erectile dysfunction. In humans, down-regulation or non-function of the leptin receptor has a prevalence of 3% in leptin receptor-mutated cohorts of early obese subjects (hetero- or homozygous individuals), which showed delayed puberty.[41] However, leptin receptor alterations are not known as major contributors to erectile dysfunction in men with erectile dysfunction resistant to treatment with phosphodiesterase type 5 (PDE5) inhibitors.[42] Therefore, the role of the leptin receptor downregulation in this mouse model is a limitation that should be taken into account both addressing signal pathways, as in the present study, and treatment aimed at reversing erectile dysfunction.[43,44]

Impairment of endothelial function is one of the main causes of diabetes-associated erectile dysfunction.[16] In db/db mice, acetylcholine relaxation of corpus cavernosum has previously been described to be reduced.[40,45] In the present study, acetylcholine relaxation was also impaired in phenylephrine-contracted corpus cavernosum strips from db/db mice compared to corpus cavernosum from db/+ and C57BL/6 control mice, suggesting high plasma glucose is associated with endothelial dysfunction in corpus cavernosum.

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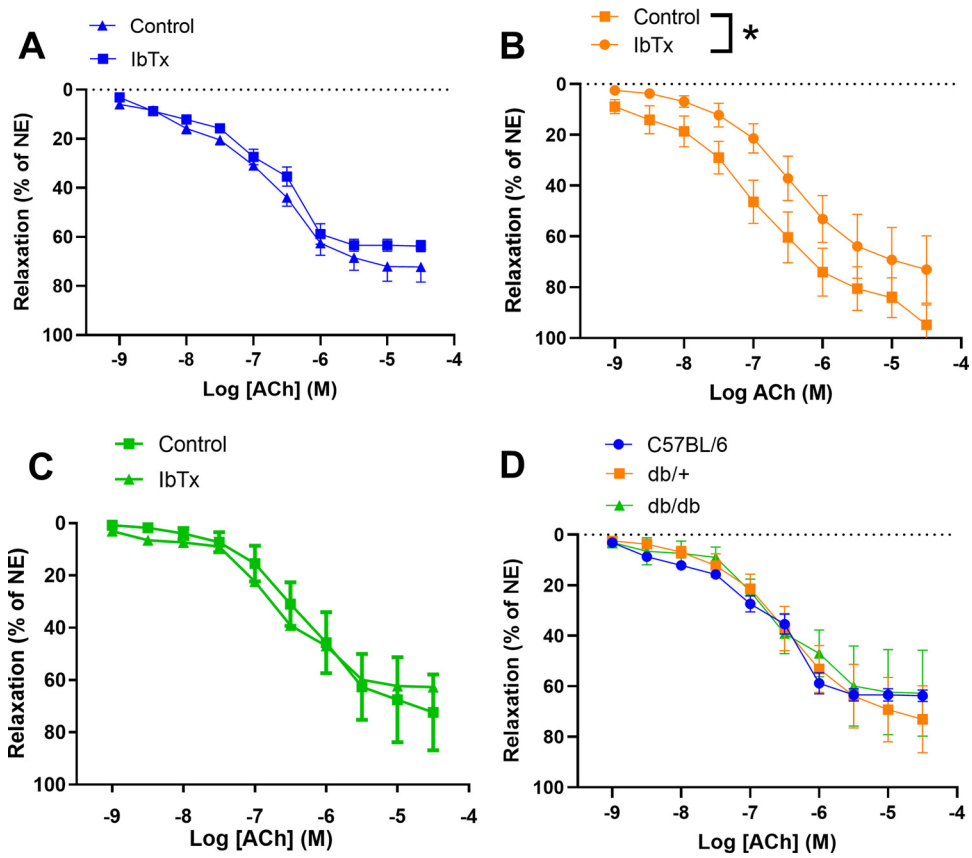

Figure 7. The function of iberiotoxin-sensitive KCa1.1 channels is decreased in corpus cavernosum from diabetic animals. Concentration-response curves for ACh (A) in the absence (n = 6) and the presence (n = 6) of iberiotoxin (IbTx 0.1 μM) in corpus cavernosum from C57BL/6, (B in the absence (n = 6) and the presence (n = 6) of iberiotoxin (IbTx 0.1 μM) in corpus cavernosum from) db/+ mice, and (C) in the absence (n = 6) and the presence (n = 6) of iberiotoxin (IbTx 0.1 μM) in corpus cavernosum from diabetic db/db mice. Results are means ± SEM * $P < .05$ vs control curve. (D) Concentration-response curves for ACh obtained the presence of iberiotoxin in corpus cavernosum from C57BL/6 mice, db/+ mice, and diabetic db/db mice. Figure is available in color online at www.jsm.jsexmed.org.

Interestingly, the impairment of acetylcholine relaxation in preparations from db/db mice was more pronounced in preparations contracted with the selective α_1-adrenoceptor agonist, phenylephrine vs the endogenous ligand, norepinephrine. In contrast to phenylephrine, norepinephrine also activates α_2-adrenoceptors in erectile tissue.[46] The response to the α_2-adrenoceptor agonist clonidine was decreased, and that may explain why concentration-response curves for norepinephrine were rightward-shifted in corpus cavernosum from db/db mice vs C57BL/6 mice. In contrast, phenylephrine contractions were increased in the corpus cavernosum from db/db mice. Therefore, altered smooth muscle contractility to α-adrenoceptor activation seems to influence the observed impairment of acetylcholine relaxation in corpus cavernosum from db/db mice. However, in addition to the altered contractility further investigation will be required to understand why the impairment of acetylcholine-induced response in phenylephrine-contracted strips seems to be more related to the glycemic state than to the leptin receptor mutation.

In human erectile tissue, NO mediates acetylcholine relaxation in the corpus cavernosum, while both NO and an endothelium-derived hyperpolarization (EDH) contribute to acetylcholine relaxation in penile arteries.[19,47] Endothelial calcium-activated K channels of small (KCa2.3) and intermediate conductance (KCa3.1) contribute to both NO and EDH-mediated relaxations in rat mesenteric arteries,[48] and relaxations mediated by these channels are impaired in mesenteric arteries from Zucker rats and db/db mice,[29,49] although the expression of KCa2.3 channels is unaltered.[29] However, mice with knockdown of the KCa2.3 channel expression develop erectile dysfunction,[35] and streptozocin-induced type 1 diabetic in rats have reduced expression of KCa2.3 and KCa3.1 channels ion corpus cavernosum.[30] In the present study, mRNA expression of KCa2.2 was decreased in corpus cavernosum of db/+ and db/db mice, while the KCa2.3 and KCa3.1 were only increased in corpus cavernosum from diabetic db/db mice (Figure 3). These increases seem compensatory and may reflect a larger protein turnover, as the protein expression of KCa2.3 was markedly decreased in erectile tissue from db/+ and db/db mice compared

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to tissue from C57BL/6 mice. Indeed, ubiquitination of misfolded protein leading to protosomal degradation of KCa2.3 and KCa3.1 channels has previously been described,[50] and it is pronounced for KCa channels in diabetes.[51] Therefore, one may speculate that the upregulated KCa2.3 mRNA coupled with increased ubiquitination of KCa2.3 protein increase KCa2.3 channel turnover in the endothelial cells of the corpus cavernosum of diabetic db/db mice.

The decreased KCa2.3 protein expression does, however, apparently not explain the impaired endothelium-dependent relaxation in diabetic db/db mice, as there were still marked differences in acetylcholine relaxation when all KCa2 channels were blocked with apamin (Figure 6D). These findings may also suggest that the functional changes correlate more with the changes in mRNA expression of the KCa2.3 than protein expression in the corpus cavernosum.

The KCa2.3 channels contribute to endothelium-dependent relaxations of corpus cavernosum in both mice and rats,[28,35] while the blocker of KCa3.1 channels, TRAM-34 does not change it.[28] The inhibitor of KCa2 channels, apamin, also markedly inhibited acetylcholine relaxations in corpus cavernosum from all 3 groups of mice in the present study suggesting KCa2 channels are involved in these relaxations. Surprisingly, in the presence of apamin, the impairment of acetylcholine relaxation was more apparent in the corpus cavernosum of db/+ and db/db mice suggesting apamin-sensitive K channels in part compensate the loss of endothelium-dependent relaxation in the corpus cavernosum of db/+ and db/db mice. These findings agree also with the observations that the opener of KCa2 and KCa3.1 channels, NS309, potentiates endothelium-dependent relaxations induced by acetylcholine to the same degree in corpus cavernosum tissue from db/+ and db/db mice.

As mentioned above, KCa3.1 channels do not appear to contribute to acetylcholine relaxation of corpus cavernosum in mice and rats.[28] However, charybdotoxin-sensitive endothelial KCa3.1 were suggested to be involved in inhibition of neurogenic contractions in rat penile arteries,[22] and in coronary arteries, the KCa3.1 channel has been described to be involved in vascular smooth muscle cell phenotype shift.[52] In the present study, the KCa3.1 mRNA expression is increased in corpus cavernosum of diabetic mice, and further investigation will be required to address whether such a phenotype shift may also take place in smooth muscle in erectile tissue from diabetic mice.

Several neurotransmitters (e.g. NO and hydrogen sulfide [H_2S]) and endothelium-derived mediators, including NO, CNP, and H_2O_2 lead to activation of KCa1.1 channels in the corpus cavernosum and penile arteries.[11,21,27,53] Moreover, knockout of the KCa1.1 channels in mice leads to erectile dysfunction and hypercontractility of the corpus cavernosum smooth muscle.[23] Openers of KCa1.1 channels potentiate erection in healthy rats,[26] and enhances the effect of the phosphodiesterase type 5 inhibitor, sildenafil, in penile arteries from diabetic patients and in type 1 diabetic rats.[54] In type 1 streptozotocin-induced diabetes in Fischer F-344 rats, diabetes caused upregulation of the channel pore-forming transcript of the KCa1.1 channels.[55] This is in agreement with our findings in db/db mice, where the KCa1.1α pore-forming transcript is upregulated compared to the C57BL/6 mice and the db/+ mice, suggesting the upregulation is due to the presence of diabetes.[55] However, at the protein level, the KCa1.1α subunit and the expression of the regulatory β subunit, which increases calcium and voltage sensitivity of the channel, were unaltered. It was previously suggested that the changes in expression and function of the KCa1.1 channels in diabetic rats are compensatory,[55] and the changes in expressions of the KCa1.1 channel subunits observed in the present study may all be a consequence of compensatory mechanisms for increased KCa1.1 channel degradation in diabetic mice. Thus, in the coronary circulation of diabetic mice, increased ubiquitination and proteosomal degradation was found for KCa1.1 channel subunits and provided as explanation of impaired endothelium-dependent coronary vasodilation.[51,56] In the present study, the blocker of KCa1.1 channels, iberiotoxin, had an inhibitory effect on acetylcholine relaxation in the corpus cavernosum of db/+ mice but lost effect in db/db mice. In the presence of iberiotoxin, acetylcholine relaxations were similar in the corpus cavernosum of all 3 experimental groups suggesting the KCa1.1 channel plays a pivotal role in the impairment of endothelium-dependent relaxation of the corpus cavernosum of diabetic mice.

Conclusion and Perspectives

Erectile function in diabetic db/db mice was severely affected compared to heterozygous and normal C57BL/6 mice. These findings suggest that the non-diabetic db/+ and diabetic db/db mice for translational purpose can be used for testing drug on moderate and severe erectile dysfunction, respectively. However, this model has the limitation that the down-regulation of the leptin receptor plays a role in the pathophysiology and will probably also influence the translation of potential findings to clinical studies. The decreased function of KCa1.1 channels compared to db/+ mice may underpin the decreased endothelium-dependent relaxation and erectile dysfunction in diabetic db/db mice. In preclinical studies, several compounds target the KCa1.1 channels[26], and gene transfer of the KCa1.1 has been attempted in a small clinical study in humans. In contrast, the development of compounds targeting the KCa2.3 channels has been limited to preclinical studies in rodents. It was suggested that KCa2.3 channels play a critical role in the repolarization of the atria of the heart and that blocking of the KCa2.3 channels may could be of potential benefit in the treatment of supraventricular arrhythmias, for example, atrial fibrillation,[57,58] although we did not find changes in ECG in healthy rats when openers of KCa2 channels were used.[28] Therefore, the effect on KCa2.3 in the heart and also blood pressure lowering effects are limitations that has to be accounted for in developing drug candidates for this target in erectile tissue.

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Corresponding Authors: Simon Comerma-Steffensen, DVM, MSc, PhD and Ulf Simonsen, MD, PhD, Department of Biomedicine, Aarhus University, Ole Worms Allé 4, Aarhus C, 8000, Denmark. Tel: 456-020-2613; E-mails: simoncomerma@biomed.au.dk us@biomed.au.dk

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STATEMENT OF AUTHORSHIP

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SUPPLEMENTARY MATERIALS

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